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Fueling the caries process: carbohydrate metabolism and gene regulation by *Streptococcus mutans*

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The nature of the oral cavity and host behaviors has mandated that the oral microbiota evolve mechanisms for coping with environmental fluctuations, especially changes in the type and availability of carbohydrates. In the case of human dental caries, the presence of excess carbohydrates is often responsible for altering the local environment to be more favorable for species associated with the initiation and progression of disease, including *Streptococcus mutans*. Some of the earliest endeavors to understand how cariogenic species respond to environmental perturbations were carried out using chemostat cultivation, which provides fine control over culture conditions and bacterial behaviors. The development of genome-scale methodologies has allowed for the combination of sophisticated cultivation technologies with genome-level analysis to more thoroughly probe how bacterial pathogens respond to environmental stimuli. Recent investigations in *S. mutans* and other closely related streptococci have begun to reveal that carbohydrate metabolism can drastically impact pathogenic potential and highlight the important influence that nutrient acquisition has on the success of pathogens; inside and outside of the oral cavity. Collectively, research into pathogenic streptococci, which have evolved in close association with the human host, has begun to unveil the essential nature of careful orchestration of carbohydrate acquisition and catabolism to allow the organisms to persist and, when conditions allow, initiate or worsen disease.

Keywords: *carbohydrate transport*; *sugar phosphotransferase system*; *dental caries*; *biofilms*; *catabolite repression*

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The capability to sequence whole bacterial genomes and the knowledge gained in the 'post-genomic' era have significantly reshaped our understanding of the etiologies and pathogenic processes of many oral diseases, including dental caries (1). For example, new information from the fields of anthropology and evolutionary genetics has provided evidence that *Streptococcus mutans* appeared in human dental plaque and that an expansion of genes important for the success of dental caries pathogens occurred around the time humans transitioned from hunter-gatherers to stable agrarian communities (2, 3). This acquisition and diversification of caries pathogens thus occurred during the period when human civilizations became dependent on foods enriched in carbohydrates from domesticated grains, which was likely a major contributing factor in the appearance of dental caries beginning in 'Post Agricultural' humans and continuing to the present (4). Studies into the oral microbiome have broadened our understanding of the diversity of organisms present in the oral cavity of modern humans, with estimates of the number of microbial taxa inhabiting this environment ranging from 600 to over 1,200 (5, 6). Analysis of the oral microbiome in healthy and in carious sites provides support for the 'ecological plaque hypothesis', which posits that specific changes to the local environment allow for cariogenic species to outcompete health-associated flora and dominate as carious lesions are initiated and progress (7). This 'ecological catastrophe' (8) leads to demineralization of tooth enamel, which is largely driven by acid production by bacteria from sugary foodstuffs introduced via the diet of the host. Cariogenic species are particularly effective at metabolizing carbohydrates to produce strong organic acids and at surviving in acidic conditions as compared to species associated with healthy dentition (8, 9). It is now well accepted that physiological processes, including carbohydrate uptake, acid generation, and the tolerance of low pH, are the major characteristics of bacteria that contribute to the development of dental caries, rather than classical virulence factors, such as secreted toxins.

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In the years since its discovery by Clarke in 1924, *S. mutans* has become the most intensively studied cariogenic organism and is the species that is most consistently associated with the initiation and progression of caries in humans (10). Studies conducted on the genetics and physiology of *S. mutans* have furthered our understanding of fundamental processes, including biofilm formation, quorum sensing, and stress responses, and how these physiological processes contribute to pathogenicity (11). Before the genomic era, major advances to our knowledge of bacterial physiology were achieved through the analysis of cell populations generated using chemostat culture, which allowed for tight control of environmental conditions and bacterial behaviors. In this review, we discuss how chemostat cultivation can be combined with genomic studies to more thoroughly assess bacterial responses to carbohydrate availability under finely controlled environmental conditions. Furthermore, as the oral cavity provides a diverse array of carbohydrate sources, we will also discuss some recent advances understanding how carbohydrate uptake and gene regulation in response to specific carbohydrates may influence persistence and virulence of *S. mutans*. We conclude with a brief discussion of how these advances are integrated with selected information about the influence of carbohydrate utilization on virulence of related pathogens.

Carbohydrate utilization by *Streptococcus mutans*

The expansion of genes related to carbohydrate uptake and metabolism appears to have been an essential evolutionary advancement contributing to the success of *S. mutans* as a caries pathogen in oral microbial biofilms (3, 12). The transport of oligosaccharides, including melibiose, raffinose, stachyose, and maltodextrans, is primarily conducted by the activity of ATP binding cassette (ABC) transporters encoded in the genome of *S. mutans*, which include the multiple sugar metabolism (*msm*) and *malXFGK* transport systems (13–15), The predominant route for uptake of mono- and disaccharides by *S. mutans* is the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The characteristics, functions, and regulation of the genes for the PTS have been reviewed extensively elsewhere (16–20). The PTS consists minimally of Enzyme I (EI) and HPr, which participate in a phosphotransfer reaction to a variety of Enzyme II (EII) permeases that concomitantly phosphorylate and internalize a spectrum of mono- or disaccharides. The genomes of certain strains of *S. mutans* encode as many as 15 EII permeases, composed of A, B, C, and sometimes D domains. Most of these permeases are harbored by the majority of strains, but there are a few that are only present in particular isolates (3, 21, 22). Undoubtedly, the diversity in carbohydrate sources that can be internalized by *S. mutans* contributes to its pathogenic potential. Recent studies have suggested that additional accessory components may be required for full functionality of certain EII complexes. For example, an open reading frame (ORF), encoding a hypothetical protein, located within the cellobiose utilization operon of *S. mutans* and *Streptococcus pneumoniae* is necessary for the efficient internalization of cellobiose (23, 24). Similarly, the *manO* gene product of *S. mutans* appears to be required for the normal function of the EII^{Man} complex (25).

Carbohydrate catabolite repression (CCR) (16, 26, 27), the process by which bacteria delay the catabolism of non-preferred carbohydrates when preferred sources are simultaneously present, is a critical process that optimizes growth and competitive fitness. Perhaps not surprisingly – given its close co-evolution with humans, dietary changes in humans over centuries and the nature of the disease caused by this organism – *S. mutans* has evolved strategies for CCR that do not completely mirror those described for many other Gram-positive organisms. In general, CCR in Gram-positives can be controlled at multiple levels. When rapidly metabolizable carbon sources are internalized, glycolytic intermediates accumulate within the cell, some of which act as allosteric activators of a kinase (HPr kinase/phosphatase) that phosphorylates HPr at serine residue 46 (16). In *S. mutans*, serine-phosphorylated HPr (HPr(Ser-P)) plays a major role in the repression of genes for the uptake and metabolism of non-preferred carbohydrate sources (28). A primary route for CCR in many Gram-positive bacteria is through the activation of the transcriptional regulator CcpA, which when allosterically activated by HPr(Ser-P) can bind to the promoter regions of multiple CCR-sensitive genes at a conserved catabolite response element (CRE). However, inactivation of the *ccpA* gene in *S. mutans* almost never results in alleviation of CCR. On the other hand, microarray studies comparing *ccpA* and wild-type *S. mutans* clearly show a critical role for this regulator in direct and indirect control of transcription of genes that encode products involved in carbon flow, energy metabolism, and the fate of pyruvate (29). In contrast to HPr and CcpA, certain EII permeases exert a dominant effect on CCR-sensitive genes in *S. mutans*, particularly when cells are grown in the presence of the sugar(s) that are internalized by these permeases (21, 30, 31). There is also another layer of control of CCR in *S. mutans*. In particular, CcpA has the ability to indirectly influence non-preferred sugar uptake by regulating the expression of multiple EII permeases (31). Overall, then, CCR in *S. mutans* appears to have evolved to allow the organism to evaluate the source and availability of carbohydrates in the environment using EII permeases while monitoring carbohydrate flow through glycolysis and global energy demands using HPr and CcpA (Fig. 1). Organization of CCR in this manner would afford *S. mutans* the ability

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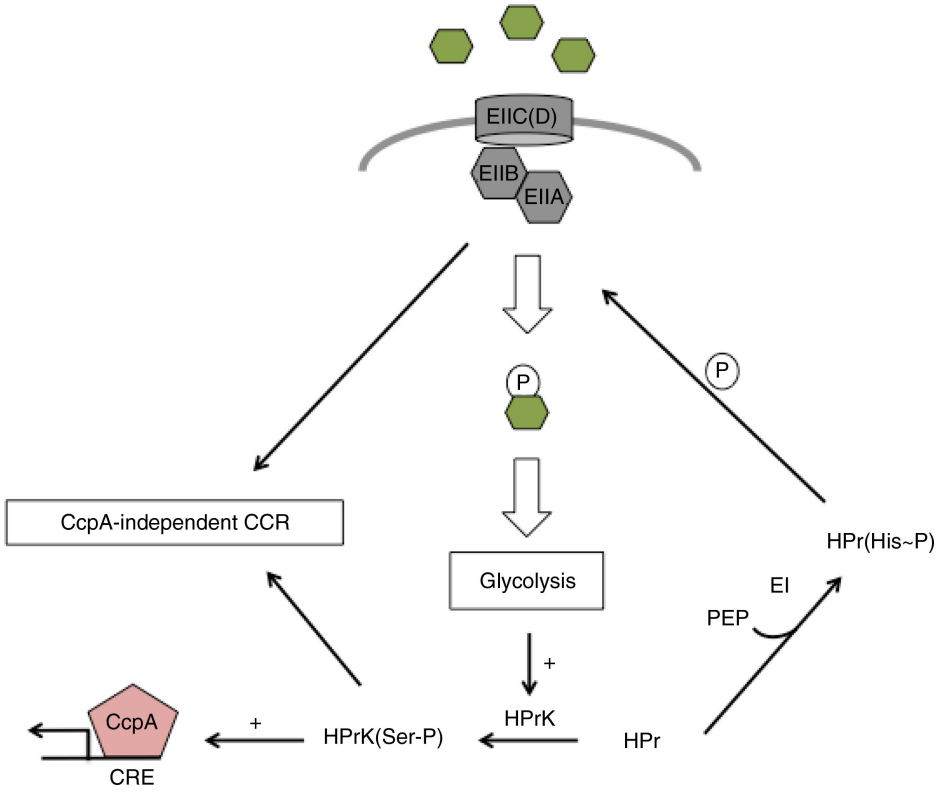

Fig. 1. *S. mutans* has evolved elegant strategies for coping with the diversity and availability of nutrients present within the oral cavity. When a preferred carbohydrate source, such as glucose, is present in abundant quantities, the elevated movement of metabolites through glycolysis profoundly alters many cellular properties. For instance, the kinase activity of the HPr kinase/phosphatase (HprK) is stimulated leading to an increase in the serine-phosphorylated species of HPr [HPr(Ser-P)]. HPr(Ser-P) acts as a co-factor of the major regulator CcpA to repress transcription of metabolic genes containing a catabolite response element (CRE) in their promoter regions, or in the cases of CcpA-independent CCR, directly affects transcription of genes associated with catabolism of non-preferred carbohydrate sources, a process that may also require certain PTS permeases. As the supply of available carbohydrate is diminished, the reduced quantity of readily metabolizable carbohydrate translates to a lower abundance of HPr(Ser-P). Without its co-factor, CcpA no longer functions as a repressor, and with lower levels of carbohydrate transport occurring, PTS porters remain predominately phosphorylated and play a less active role in CCR. Relief of repression by these factors allows for transcription of a variety of genes, including PTS porters, for sensing and accessing a diverse array of carbohydrate sources. Overall, PTS porters monitor the type and levels of carbohydrates present in the oral cavity, while the overall supply of carbohydrate in relation to cellular energy demands is monitored by the activity of HPr and CcpA.

to adjust carbohydrate uptake and metabolism pathways rapidly and seamlessly in response to the ever-changing environment within the oral cavity.

Continuous culture as a valuable tool for analyzing bacterial physiology

Since its development (32, 33), chemostat cultivation has aided in the discovery and characterization of many processes related to microbial physiology, and additional opportunities for this technology remain in the 'post-genomic' era (34). Chemostat cultivation provides fine control of multiple variables that impact bacterial physiology and gene expression, including pH, temperature, growth rate, atmospheric composition, and the source and availability of specific nutrients. Continuous culture has proven to be extremely valuable to researchers seeking to unravel the impact of the availability of particular nutrients on the metabolic pathways of bacteria, including the mutans streptococci. Information on specific aspects of theory and design of continuous culture can be found elsewhere (34, 35).

The saccharolytic metabolism of lactic acid bacteria, including *S. mutans* and many other oral streptococci, makes these bacteria highly amenable to chemostat cultivation, as the organisms do not respire and fermentation of carbohydrates is the overwhelmingly dominant source of energy for growth and maintenance. In early studies of *S. mutans*, chemostat culture was exploited to determine how perturbations to the environment impacted the composition of the bacterial envelope, especially the lipoteichoic acids (LTAs) (36–41). As it was established that attachment to the tooth pellicle and the construction of extracellular polysaccharides by the activity of glucosyltranferases (GTFs) were critical for the success of

S. mutans as a pathogen, a variety of chemostat studies were initiated to characterize these virulence factors, and they revealed that the production, activity, and localization of the GTFs were greatly influenced by growth rate, carbohydrate source, pH and other factors (42–45). Another major aspect contributing to the success of *S. mutans* in oral biofilms is its ability to uptake a variety of carbohydrate sources and alter its metabolism in response to the local microenvironment, and chemostat studies have greatly aided in our understanding of how environmental inputs impact PTS activity and the levels and phosphorylation state of various component of the PTS (46–53). In addition to the findings mentioned above, the chemostat model has been instrumental in shaping our understanding of the impact of various environmental stimuli and growth rate on fermentation end products (54), acidurance (55, 56), membrane fatty acid content (57) and the effects of trace metals on growth (58–60), among other critical processes. Collectively, these studies provided a firm foundation of how the basic physiological and virulence-related properties of *S. mutans* are influenced by environmental alterations.

Though batch culture is the overwhelming method for studying *S. mutans* genetics and physiology, the chemostat model is still utilized in low throughput studies of *S. mutans* where tight control of growth parameters is crucial. Some recent examples include explorations of the proteome of *S. mutans* in response to selected growth conditions, including an acidic environment (61, 62), of the GlnR-mediated response to low pH (63) and of the contribution of NADH oxidase to acid and oxidative stress (64). As genomic technologies continue to develop, prior knowledge generated can guide the design of new studies that merge continuous culture techniques with genome-scale methodologies to further broaden our understanding of the adaptive capabilities of this caries pathogen.

In order to integrate observations of physiological responses to carbohydrate availability with transcriptional methodologies, we conducted a continuous culture study of the reference strain *S. mutans* UA159 and a mutant lacking the $EIIAB^{Man}$ permease, *manL*, grown in base medium formulated with either 10 mM glucose, which was determined to be glucose limiting, or 100 mM glucose, which supplied glucose in excess (65). Multiple parameters of the PTS were probed in the *manL* mutant and parental strains to demonstrate that UA159 responds similarly to previously characterized strains of *S. mutans*. Most notably, in pH drop experiments, cells of the parental strain grown in limiting carbohydrate lowered the pH faster and reached a lower terminal pH upon addition of exogenous glucose, compared with cells grown in excess glucose (65). In pH drop assays, cells halt metabolism due to the acidification of the cytoplasm and the acid-sensitive nature of the PTS enzymes, not because they have exhausted the supply of fermentable carbohydrate (55). Thus, cells able to achieve a lower terminal pH are generally regarded as more acid tolerant. Though there is evidence for the presence of a proton-glucose symporter, which is active in PTS-deficient cells (66), it is likely that under conditions of low pH the majority of carbohydrates are transported via residual PTS activity. A small number of sugars may also be transported by ABC transporters under these conditions.

To test the relative levels of intracellular glycogen-like energy stores, chemostat cultivated cells were also assayed for their ability to lower the pH without the addition of exogenous carbohydrates. It was found that cells grown in excess glucose produced a lower final pH than cells grown in limiting carbohydrate, indicative of the accumulation of higher levels of intracellular glycogen-like energy stores (65), closely resembling previous results (46). In the same study (65), the PEP-dependent transport of glucose, mannose and fructose was found to be lower in both the *manL* mutant and parental strains when cultured in excess versus limiting glucose, and in relation to the parental strain, the *manL* mutant displayed diminished transport of all sugars tested, but particularly of glucose and mannose (65). The protein levels of the general PTS proteins EI and HPr in the *manL* deletion and parental strains experienced very little change between limiting and excess carbohydrate conditions (65), and these observations are generally consistent with observations with batch-grown cells (67) and with chemostat cultivated *S. mutans* grown under various conditions (47–49). Finally, it was noted that under excess glucose conditions there was a higher abundance of both the HPr(Ser-P) and HPr(Ser-P)(His ~P) forms of HPr in both the *manL* and parental strains, while under glucose limitation, the unphosphorylated and histidine-phosphorylated species predominated. These results were similar to what has been noted with batch-grown cells (68). Further, in continuous culture studies under increasing concentrations of glucose, availability of the HPr(Ser-P) and HPr (Ser-P)(His ~P) forms of HPr was generally increased in *S. mutans*; with a corresponding decrease in the abundance of unphosphorylated and histidine-phosphorylated forms (69). Thus, growth in glucose replete conditions strongly favors serine phosphorylation of HPr, which can then serve as a repressor for the catabolism of more complex carbohydrates or non-preferred mono- and disaccharides (28). Collectively, studies of the regulation of PTS components in *S. mutans* have generally concluded that perturbations in the environment can strongly influence the availability and activity of EII enzymes. However, the absolute levels of the general PTS proteins do not fluctuate to nearly as great an extent in response to environmental inputs, albeit the phosphorylation status of HPr appears very sensitive to environmental conditions.

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Physiologic analysis of the effects of carbohydrate availability on *S. mutans* UA159 is complex and attributes that are directly correlated with virulence potential are affected. Thus, a transcriptional study of *S. mutans* UA159 grown in limiting versus excess glucose was conducted using microarray techniques (65). Under conditions of glucose limitation, cells experienced a derepression of transporters associated with carbohydrate uptake, as evidenced by the elevation of several PTS EII enzymes and the genes for the *msm* operon. Genes involved in glycogen catabolism, energy metabolism, and pyruvate fate, including pyruvate dehydrogenase, were also elevated. Under glucose excess, where cells are likely limited for amino acids or a group of amino acids, we found that many transcripts for biosynthesis of amino acids, nucleotides, and cofactors were upregulated, as well as several ABC transporters, some of which were specific for the internalization of amino acids. Interestingly under glucose limitation, the *ccpA* transcript and many genes known to be part of the CcpA regulon were found to be upregulated, while the global transcriptional regulator CodY and several genes known to be under its control were upregulated when glucose was present in excess. From these observations, it was concluded that the coordinated activity of CcpA and CodY might be an important regulatory mechanism in cellular responses to alterations in carbohydrate availability and amino acid limitation.

Other methods for analyzing the impact of carbohydrates on bacterial physiology

Chemostat cultivation serves as an excellent method for obtaining reproducible data evaluating the impact of environmental perturbations on pure populations of planktonic bacteria. However, in the oral cavity, bacteria grow in multispecies biofilms where diffusion limitation and other factors lead to populations that are heterogeneous in terms of their microenvironments and their growth rate and growth phases. In addition, saliva and the rate of salivary flow contribute greatly to the microbial ecology of the oral cavity and the severity of dental caries, as well as other oral diseases. With these considerations in mind, we will briefly discuss several other models of oral microbial communities and how dietary carbohydrates contribute to the success of pathogens under these conditions.

Biofilm models of bacteria have been developed to monitor the formation of biofilm communities and their responses to environmental variables, such as nutrient type and availability. In addition to simple mono- and disaccharides, oral microbial biofilms likely encounter many structurally complex carbohydrate sources, including polysaccharides, such as starch, that are introduced via the diet or host-derived glycoproteins such as mucins, and these diverse carbohydrates can have a profound impact on bacterial physiology and virulence. In a recent study addressing the impact of complex carbohydrates on microbial biofilms, Klein et al. monitored the influences of starch and sucrose in the presence of host-derived amylase on the transcriptome and physiology of *S. mutans* in a biofilm setting (70). Importantly, bacteria were found to up-regulate genes associated with the transport and metabolism of maltose and maltotriose at early timepoints, indicating that the bacteria could access the sugars released via amylase (70). Further, an increased expression in genes related to glycogen metabolism and in the amount of glycogen detected in biofilms, as a result of addition of starch led the authors to propose that *S. mutans* could benefit from the slow release of nutrients from starch (70). Of particular interest, in the presence of starch and sucrose, the authors noted the upregulation of genes encoding proteins contributing to stress tolerance, including *groEL* and *groES* along with the osmoresponsive *pacL* and *trkB* genes (70). Similarly, when readily fermentable carbohydrates become limiting in the oral cavity, it is likely that microbial biofilms access host-derived glycoproteins for nutrients, including mucins. Though studies have shown that *S. mutans* cannot grow well with mucin as a sole carbon and energy source (71), when mucin was added to sucrose-starved biofilms, the bacteria displayed enhanced survival (72).

Human saliva serves a primary role in maintaining the health of oral sites, and the unique proteins and finely tuned buffering capacity of these fluids greatly impact the composition of the oral microbiota, in many cases preferentially selecting for the survival of non-cariogenic organisms (73). The utilization of human saliva in models of dental caries is often limited due to the difficulty of obtaining sufficient volumes of material for experiments. In some instances, these challenges have been overcome by the creation of artificial saliva, and though not as complex as saliva obtained from human subjects, the results obtained with this medium are in some cases comparable to those of human saliva (74, 75). As described by van der Hoeven and co-workers, *S. mutans* cannot survive with mucin as a sole source of carbohydrates (71) and fares much better in artificial and human saliva supplemented with carbohydrates (75). This requirement for supplemental carbohydrates in saliva is further illustrated in classical studies of *S. mutans* in continuous chemostat cultivation with artificial and human saliva, which noted an overall lower recovery of the organism in the absence of supplemented fermentable carbohydrates (76). Further, in studies of human subjects, a decrease in the presence of *S. mutans* has been observed when carbohydrates were removed from the diet with a reemergence of the organism when the consumption of carbohydrates was resumed (77). The nutrient content and ionic strength of saliva is quite different from the components of complex media sources typically utilized in the laboratory. It is however sometimes necessary to utilize rich and defined media

during experimentation in order to ensure the vigorous growth of the organism under study while assessing the characteristics of interest.

When approaching the topic of carious lesions from the perspective of preventing or assessing damage to odontological tissues, experiments utilizing animal models and studies of populations of humans have been particularly useful for advancing our understanding of the role that carbohydrates play in the initiation and progression of disease. A most helpful review discussing the utility of the rat caries model and its importance in the study of dental caries was recently written by Bowen (78). In this review, Bowen describes several instances where the rat caries model has shaped our understanding of critical concepts related to dental caries. For example, studies of germ free and antibiotic-treated rats were instrumental in demonstrating that the presence of microbes within the oral cavity is critical for the development of carious lesions. In addition, studies using rats have illustrated that it is not as much the consumption of particular sugars but the frequency of consumption that strongly contributes to caries, and further, many anti-caries treatments succeed mostly due to their impact on the frequency of meals (78). Similar results have been observed in human studies, and as presented in a review by Bradshaw and Lynch, the frequency and amount of sugar, particularly sucrose, consumed by human subjects is strongly linked to the development of dental caries (79). The results generated from early human studies precipitated the original, perhaps oversimplified, implication of sucrose as 'the arch criminal of dental caries' (80) and *S. mutans* as the causal organism. Further studies of the physiology and cariogenic potential of oral microbial pathogens have altered our understanding of how carious lesions originate and modified the strategy for preventing carious lesions from directly eliminating carious bacteria from the oral cavity to reestablishing a balance in the ecology between health and caries associated flora (8). Similarly, though sucrose does hold a strong causal link to caries, research conducted on the association of other carbohydrates sources with caries formation continues to broaden our view (81). Additional evidence suggests that other dietary carbohydrates, including particularly high concentrations of starch, can also result in caries due notably to the retention of these foodstuffs in the oral cavity (79). These observations lead us to the conclusion that though sucrose is critical to our understanding of caries etiology, it must also be considered that other sugars may still play important roles (81).

Recent progress on effects of carbohydrate source

Oral microbial biofilms are exposed to a vast array of carbohydrate sources that are provided via the diet or host secretions, or that are generated by the oral flora. As the nutrient source can significantly influence the composition and pathogenic potential of oral biofilms, it is important to determine how various carbohydrate sources are internalized and metabolized. Further, the diverse repertoire of PTS permeases encoded in the genome of *S. mutans* significantly contributes to its survival and cariogenic potential. The advent of genome-scale technologies has greatly enhanced our ability to study the influence of various nutrient sources on the transcriptome of organisms, and a major leap in our understanding of the impact that carbohydrate sources have on gene expression in *S. mutans* was achieved in a systematic analysis of transcriptional changes that occur in response to a variety of mono- and oligosaccharides using microarray methodology (82). This study clearly illustrates how discrete alterations to the transcriptome can occur as a result of changes in nutrient type. In the following section, we will focus our discussion on recent studies that delve into mechanistic aspects of the control of the metabolism by a select group of carbohydrate sources and how these processes may be integrated with the virulence of *S. mutans*. Rather than present an exhaustive discussion of all carbohydrate sources, we have selected certain carbohydrates that are regularly encountered by *S. mutans* in the oral cavity where significant progress in understanding the mechanistic aspects of metabolism has recently been made.

Sucrose

Sucrose, a β2,1-linked disaccharide of fructose and glucose, remains a major constituent of the human diet and is strongly linked to the initiation and progression of dental cares. *S. mutans* produces multiple exoenzymes capable of acting on sucrose. These include the glucosyltransferase enzyme GtfB (sometimes referred to as GTF-I), which acts on sucrose to produce water-insoluble glucans composed predominantly of α1,3-linkages, and GtfD (sometime called GTF-S) that converts sucrose to mostly soluble α1,6-linked glucans. An additional enzyme GtfC (sometimes GTF-SI) makes a glucan with mixed α1,3 and α1,6 linkages (83–85). The high molecular weight polysaccharides produced by the GTF enzymes have been implicated in attachment and biofilm formation by *S. mutans* on the smooth surfaces of the tooth (86). In addition, a fructosyltransferase (FTF) enzyme acts on sucrose to produce a fructose homopolymer that is primarily composed of β2,1-linked fructose (inulin). Unlike *S. mutans*-derived glucans, fructans serve mainly as an extracellular storage polymer (87). To access fructose polymers, *S. mutans* produces a secreted exo-β-D-fructosidase, FruA, that releases fructose from β2,6– and β2,1-linked fructan polymers (88, 89). FruA can also act as an invertase, cleaving extracellular sucrose into fructose and glucose. At the surface of the cell, the PTS enzyme EII^{Scr}, encoded by the single gene *scrA*,

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phosphorylates and internalizes sucrose. In fact, the majority of sucrose encountered by *S. mutans* is internalized and metabolized to produce organic acids, with a relatively minor portion being converted to extracellular polymers (90), at least in *in vitro* studies.

Though carbohydrate transporters other than ScrA have been implicated in the internalization of sucrose, it has been challenging to determine the exact contribution of the PTS and/or ABC transporters to sucrose uptake because of the presence of the many sucrolytic exoenzymes produced by *S. mutans*. To address this problem, a variety of strains containing mutations in some or all of the genes for sucrolytic enzymes, including *gtfA, gtfBC, gtfD, ftf,* and *fruA*, were created in the *S. mutans* UA159 genetic background (30). Through growth studies conducted using these mutants, it was demonstrated that metabolism of sucrose in planktonic *S. mutans* cultures is primarily attributable to the activity of the exoenzymes GtfBC that release free fructose as they generate glucans, and to the PTS enzyme EII^{Scr}, It was also observed that the exoenzyme GtfD played a minor role in the metabolism of sucrose, and little contribution to extracellular sucrose hydrolysis was noted for FruA, although this is probably because the *fruA* gene was poorly expressed under the conditions tested. Another question addressed by this study was what role major carbohydrate uptake systems had on the transport of sucrose in the absence of sucrolytic enzymes. It was demonstrated that the primary PTS permease (EII^{Tre}) for trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) and the *msm* ABC transporter contributed modestly to growth on sucrose, so these systems appear to function in lower-affinity sucrose uptake, as suggested previously (14, 91). Further, it was also possible to assess the PTS-dependent transport of sucrose in this study without the confounding influence of the sucrolytic exoenzymes, and the results clearly showed that EII^{Scr} was the major route for transport of sucrose, whereas EII^{Tre} was capable of only modest levels of transport (30). Also of note, the *msm* system was shown by microarray analysis to display lower expression when *S. mutans* was growing in sucrose as compared to glucose (82). Conversely, expression of *scrA* was shown to be constitutively high under all carbohydrate conditions tested (82), perhaps indicative of evolutionary pressure for *S. mutans* to be capable of assimilating sucrose quickly when it appears in the diet.

Additional evidence presented in the report by Zeng et al. demonstrated that EII^{Scr} could also exert effects on sucrose metabolism by influencing the expression of *levD* (the EIIA component of a fructose PTS), *fruA,* and other genes (30). First, loss of *scrA* was shown to result in elevated expression of *fruA* and improved growth on the fructooligosaccharide, FOS. This phenotype could implicate EII^{Scr} in substrate-dependent CCR, perhaps acting through HPr and bypassing the CcpA circuit. Second, loss of *scrA* caused loss of sucrose-dependent activation of the LevQRST pathway. The LevQRST four-component system activates *fruA* and *levDEFG* expression in response to extracellular signals, including the presence of fructose and mannose, and is sensitive to CcpA-independent catabolite repression (31, 92). Using mutants lacking sucrolytic exoenzymes, it was demonstrated that activation of the *levD* promoter could be achieved by pulsing the strain with increasing concentrations of sucrose. However, deletion of EII^{Scr} in strains lacking multiple sucrolytic enzymes resulted in loss of *levD* promoter activation, as did the deletion of major components of the LevQRST system (30). These results could be explained as EII^{Scr} directly participating in LevQRST signaling, with the potential to fine-tune gene regulation in response to the sucrose concentration in the environment. Alternatively, it was suggested that fructose-expulsion resulted from sucrose metabolism by EII^{Scr} and ScrB, a sucrose-6-phosphate hydrolase that cleaves the internalized product of EII^{Scr}. This hypothesis was corroborated when significant concentrations of free fructose ($\sim$100 μM) were found to be released by cells lacking GtfABCD, FTF, and FruA, but not ScrA, following pulsing with 10 mM sucrose (Zeng and Burne, unpublished data). Thus, the use of a suite of mutants has helped reveal important contributions of various sucrolytic systems to regulation of *S. mutans* gene expression and ScrA is clearly an important contributor to CCR when sucrose is present.

More recently, a new PTS transport system relevant to sucrose utilization was identified. In particular, it was determined that previously unannotated genes SMU. 100–105 in *S. mutans* UA159 showed enhanced expression when cells were cultivated in biofilms in the presence of sucrose, but not when glucose or fructose were provided for growth in biofilms (93). Encoded in this gene cluster was a permease designated as PTS^{Bio}. Interestingly, it was noted that optimal expression of these genes was dependent upon the presence of functional GtfB and GtfC and that PTS^{Bio} was capable of internalizing the α1,3-linked disaccharide nigerose. Thus, PTS^{Bio} appears to transport oligosaccharides that could arise from the action of the GTFs (93), while other genes in the cluster are required for catabolism of the sugars. Clearly, then, great progress has been realized recently on understanding the complexities of the biochemistry and genetics of sucrose dissimilation, although additional work will be required to fully understand the influence that sucrose has on *S. mutans* physiology and virulence potential.

N-*Acetylglucosamine/glucosamine*

N-Acetylglucosamine (GlcNAc) and the deacetylated version of the sugar glucosamine (GlcN) are among the most abundant carbohydrates on the planet. Found in biological structures as diverse as the shells of arthropods to the cell walls of fungi and bacteria, these amino sugars

could serve as attractive nutrients for *S. mutans,* as they provide a source of both carbon and nitrogen. Researchers in the field of oral biology have long known that *S. mutans* is capable of utilizing both GlcN and GlcNAc, and GlcNAc has classically been utilized as a method for distinguishing *S. mutans* from the related cariogenic species *Streptococcus sobrinus* (94, 95). Early studies using the sugar analog streptozotocin led to the conclusion that GlcNAc enters *S. mutans* through a relatively high-affinity porter as well as through a lower-affinity permease (96), though the identity of these systems was not determined. Recently, it was found that import of GlcNAc in *S. mutans* occurs predominantly through the glucose/mannose EII enzyme complex (EII^{Man}) as *N*-acetylglucosamine-6-phosphate (25), which is converted to glucosamine-6-phosphate by the activity of the enzyme *N*-acetylglucosamine-6-phosphate deacetylase (NagA). Subsequently, glucosamine-6-phosphate can be used directly for cell wall biosynthesis or acted on by the enzyme glucosamine-6-phosphate deaminase (NagB) to produce fructose-6-phosphate, followed by entry into the Embden–Meyerhof–Parnas pathway. The EII^{Man} complex also serves as the primary mechanism for internalizing GlcN, which generates glucosamine-6-phosphate, although it appears that residual transport by other permeases, including the cellobiose and fructose permeases, contributes to internalizing GlcN (25). When cells require glucosamine-6-phosphate for cell wall biosynthesis and exogenous sources are unavailable, glucosamine-6-phosphate can be synthesized *de novo* using free fructose-6-phosphate and glutamine by glucosamine-6-phosphate synthase (GlmS).

Early investigations into the metabolism of GlcNAc by oral streptococci demonstrated that *S. mutans* predominated when grown in a mixed culture with *S. sobrinus* in medium containing a combination of glucose and GlcNAc as the primary carbohydrate sources, and this was attributed in part to the fact that *S. mutans* produced higher activity of the NagA and NagB enzymes than *S. sobrinus* (97). Recent findings regarding the utilization of GlcNAc by *S. mutans* UA159 have demonstrated that the levels of *nagB* and *glmS* mRNA, as well as the levels of the enzymes in the cell, are inversely related (98). NagB levels in the cell were elevated as the concentration of GlcNAc in the media was increased, whereas GlmS levels decreased under the same conditions. Further, a mutation of *nagB* resulted in an inability to grow in the presence of GlcNAc, a decrease in biofilm formation and the diminished levels of virulence-associated proteins, including GtfBC and PAc. Loss of *glmS* resulted in cells being unable to grow in the absence of GlcNAc and displaying an increase in the expression of GtfBC and biofilm formation (98). Control of production of these enzymes in this manner would prevent metabolism of GlcNAc when needed for anabolic processes.

The regulation of amino sugar catabolism has been well characterized in several notable organisms (99–102). In the case of *S. mutans*, transcription of *nagA* and *nagB*, which are located within separate operons, is under the control of a GntR/HutC-type transcriptional regulator, NagR, which binds near the *nagA* and *nagB* promoter (25). Initial functional studies conducted *in vitro* showed that the NagR protein of *S. mutans* could be allosterically regulated by glucosamine-6-phosphate (25). Surprisingly, it was also found that NagR is capable of binding to the promoter region upstream of *glmS* and has a direct role in regulating its transcription. Past reports have demonstrated that regulation of *glmS* is controlled by small RNAs (sRNAs) in *E. coli* and other members of *Enterobacteriaceae* (103, 104), or by a metabolite-binding ribozyme in many Gram-positive organisms (105, 106). Analysis of the genomes of multiple Gram-positive species has revealed that the canonical residues for the formation of the ribozyme structure are notably absent in species of streptococci and most species in the genus *Lactobacillus* (98, 105). Our working hypothesis is that differential regulation of *nagA*, *nagB* and *glmS* is mediated through NagR in response to various pools of metabolites via allosteric regulation; however, the possibility remains that additional currently unknown regulators exist to ensure proper balance of NagAB and GlmS levels and/or activity.

Galactose

Galactose is an epimer of glucose and is common in dairy products and on the surface of many microbial cells and host proteins. The import of galactose by *S. mutans* strain UA159 is conducted primarily through low-affinity transport via the activity of the glucose/mannose (EII^{Man}) and lactose PTS permeases (107). Within the cell, galactose-6-phosphate enters either the Leloir or tagatose pathways for further catabolism. Early co-culture studies of *S. mutans* UA159 and the oral commensal *Streptococcus gordonii* DL1 demonstrated that *S. gordonii* can effectively outcompete *S. mutans* due to the presence of a high-affinity galactose PTS, a trait that may help to promote dental health (108, 109). However, a recent analysis of the genome sequences of 57 isolates of *S. mutans* from around the world (3) revealed that a predicted galactose-specific PTS system is present in the genomes of 35 of the 57 isolates (21). It was then shown that these strains did in fact produce a high-affinity PTS transporter of galactose that was inactivated when the genes were mutated. Also of note, a strain harboring the galactose PTS system was more effective at competing with *S. gordonii* in a mixed co-culture experiment and lowering the pH than an otherwise-isogenic mutant lacking this system (21). As galactose is generally considered a non-preferred carbohydrate, the presence of this high-affinity galactose PTS system could serve to enhance the cariogenic potential

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of *S. mutans* by adding to its repertoire of preferred carbohydrates. However, another interpretation of these results is that there is a divergence among isolates of *S. mutans*, with one group of bacteria that underwent a niche adaptation that favored the acquisition or retention of high-affinity galactose transport and one that did not. One could postulate that perhaps more-cariogenic species lost the galactose transporter, whereas those that retained the system are better adapted to co-existence with commensals in less-cariogenic biofilms or in sites in the mouth that may be enriched for galactose, e.g. sites near mucus-secreting salivary glands.

One abundant source of galactose in the oral cavity is mucin glycoproteins. A recent study on the long-term survival of *S. mutans* UA159 demonstrated that the addition of mucin to a medium containing sufficient quantities of amino acids significantly enhanced the survival of *S. mutans*, and this benefit required the presence of an intact tagatose pathway (110), which is a primary route for galactose catabolism in *S. mutans.* This study provides an interesting example of how *S. mutans* may exploit mucin as a supplementary nutrient source during fasting periods by the host, though not as the sole carbon and energy source (71). As the highly cariogenic reference strain UA159 possesses only the glucose/mannose and lactose PTS for galactose transport, it remains to be seen what effect the presence of a high-affinity galactose permease in *S. mutans* might have on the long-term survival of this organism in the presence of galactose-containing glycoproteins, such as mucin. Another issue that remains unresolved is whether *S. mutans* strains possess glycosidases that can effectively access galactose in glycoconjugates or if other members of the oral microbiota provide these activities in *trans.*

Cellobiose

As discussed above, the regulation of CCR in *S. mutans* is not dominated by CcpA, and this is particularly notable for catabolism of the β-glucoside, cellobiose, which is composed of two glucose moieties joined by a β1,4 linkage (4-β-D-glucopyranosyl-D-glucopyranose). Genetic analysis of the *cel* operon has revealed that cellobiose is internalized by the $EIIC^{Cel}$ permease (*celD*) and phosphorylated by the activity of $EIIA^{Cel}$ (*celC*) and $EIIB^{Cel}$ (*celB*) (111). The internalized disaccharide can then be acted on by the activity of a phospho-β-glucosidase enzyme encoded by *celA*. The deletion of *celA* or *celD* in *S. mutans* UA159 resulted in a strain that was unable to grow in the presence of cellobiose, which indicated that the EII^{Cel} complex is the exclusive system for the transport of cellobiose and that there are no exoenzymes expressed under the growth conditions tested that can cleave cellobiose to release free glucose (111). The genes for cellobiose utilization are regulated by CelR, which contains two DNA-binding domains at the N-terminus, two PTS regulatory domains (PRD) (112) in the center of the protein and one EIIA-like domain at the C-terminus. Five of the histidine residues in CelR are predicted to undergo phosphorylation, two located in each PRD (H226 and H284; H332 and H391) and one (H576) located in the EIIA-like domain. The primary structure of CelR is similar to the mannitol transcriptional regulator MtlR of *Geobacillus stearothermophilus* (24, 113–115). Microarray studies comparing a strain lacking the *manL* gene for the glucose/mannose EIIAB protein revealed that *celA*, *celC,* and *celD* were highly expressed in the *manL* mutant when compared to the wild-type strain (116). Also of note, a *ccpA* mutant did not display a similar upregulation of the *cel* operon when assayed by microarray (29), though a subsequent RNA-Seq study did show modest elevation in *cel* operon expression upon deletion of *ccpA* (117).

In order to determine the contribution of ManL, certain other PTS components and CcpA to cellobiose utilization, a study characterizing the genetic regulation of the *cel* operon was initiated (24). It was demonstrated that CelR is required to activate the *cel* operon through a mechanism dependent on the PRD domains located within CelR. Mutation of PRD histidine residues 284 or 391 to alanine resulted in constitutive activation of the *cel* operon, whereas similar mutations of histidine residues 226, 332 or 576 resulted in a loss of *cel* operon expression. Further testing showed that with regard to CelR activity, the latter three histidine residues were dominant to the former two, and it was interpreted that histidine residues 226, 332, and 576 are targets for the general PTS proteins EI and HPr, and that phosphorylation of these residues is required for activation of CelR (24). The loss of $EIIA^{Cel}$ led to constitutive expression of *cel* genes and an $EIIB^{Cel}$ deletion drastically, although not completely, reduced the ability for cellobiose to activate *cel* genes. Initially, the former result was interpreted as a requirement of phosphorylation at histidine residues 284 and 391 by $EIIA^{Cel}$ to deactivate CelR, which was removed with the loss of $EIIA^{Cel}$. Likewise, deletion of $EIIB^{Cel}$ was thought to cause constitutive phosphorylation of $EIIA^{Cel}$, leading to inactivation of CelR (24). Notably, lack of $EIIC^{Cel}$, which should also lead to constitutive phosphorylation of $EIIA^{Cel}$, resulted in complete loss of *cel* gene activation in the presence of cellobiose. In a recent study of the homologous PRD-containing regulator MtlR of *Bacillus subtilis* (118), it was reported that membrane sequestration of MtlR by a dephosphorylated $EIIB^{Mtl}$ is essential for full functionality of MtlR and the subsequent activation of downstream catabolic genes. This observation with MtlR could provide a possible explanation for the behavior of *S. mutans* UA159 lacking $EIIB^{Cel}$, whereby phosphorylation by $EIIB^{Cel}$ serves to inactivate CelR, and dephosphorylated $EIIB^{Cel}$ serves as a tether to keep CelR associated with the membrane and facilitates *cel* operon activation.

However, it remains to be experimentally proven if CelR of *S. mutans* is regulated in this fashion.

Another level of regulation seen for cellobiose utilization involves the EII permeases enzymes ManL, FruCD, and LevD. Deletion of *manL* results in upregulation of the *cel* operon, and the most profound derepression occurs in the presence of glucose, as it serves as a secondary substrate of EII^{Cel}. Enhanced expression of the *cel* operon in the presence of the repressing sugars fructose or mannose required deletion of multiple fructose and mannose porters, i.e. *manL*, *fruCD*, *fruI,* and *levD*, to become apparent. The authors concluded that ManL, in the presence of glucose, siphons PEP-derived phosphate groups from CelR, which results in decreased expression from the operon (24). In this manner, the PRD-containing transcription regulator CelR adds another dimension to the picture of CcpA-independent CCR in *S. mutans* and provides further examples of the profound influence that EII permeases can have on metabolism of preferred and non-preferred carbohydrates.

Impact of carbohydrate metabolism in other pathogenic streptococci

When considering bacterial pathogens of humans, a great deal of attention has been placed on the production of virulence factors that mediate the destruction of host cells, such as secreted exotoxins. However, a critical area of study, often neglected, is the requirement by invading pathogens to acquire nutrients both during asymptomatic carriage and while eliciting pathologies. Not surprisingly, carbohydrate utilization pathways are intimately tied to colonization and the production of virulence compounds in many pathogens, including many bacteria that are related to *S. mutans*. As we conclude this discussion of carbohydrate metabolism and how it may influence pathogenesis, we will highlight certain recent advances in carbohydrate utilization and catabolite repression in the human pathogens *Streptococcus pyogenes* and *S. pneumoniae* and how these studies provided interesting perspectives on concepts discussed above.

S. pyogenes is capable of eliciting diseases in a diverse range of sites across the human body, including the skin and deeper tissues, the respiratory tract and in extreme cases, the bloodstream. Recent studies have begun to illustrate the critical importance of nutrient acquisition to the success of this pathogen. For example, the latest study of the general PTS proteins, EI and HPr, of *S. pyogenes* has implicated EI (*ptsI*) in the regulation of the *sag* operon, which produces the streptolysin S (SLS) toxin (119), while HPr is considered absolutely required for growth; as has been demonstrated in *S. mutans* as well as *S. pneumoniae* (28, 119, 120). In addition to the role played by the general PTS proteins, three carbohydrate responsive regulators in *S. pyogenes* have recently been shown to be critically important to the pathogen's success. Firstly, CcpA plays an important role in coordinating virulence gene expression, and deletion of *ccpA* has in some cases been linked to a loss of virulence in murine models of infection (121, 122). Other researchers have observed an enhancement of virulence in the *ccpA* deletion strain similarly to that exhibited by the *ptsI* mutant though less severe (123).

A second nutrient responsive regulator of *S. pyogenes* is LacD.1, a tagatose-1,6-bisphosphate aldolase, which is encoded in one of two *lac* operons located in the *S. pyogenes* genomes (124, 125). Interestingly, regulation by LacD.1 is independent of the enzyme's catalytic activity though likely dependent on substrate binding. Instead, LacD.1 is primarily believed to serve as a metabolite-sensing co-regulator of enzymes including the DNA-binding regulator Rgg (also called RopB) (124, 125). *S. mutans* UA159 has also been noted to contain a functional *lacD* gene (126–128) and an uncharacterized, orthologous gene *lacD2* (Abranches et al., unpublished results). Investigations into the role of nutrient-sensing regulators in *S. pyogenes*, including CcpA and LacD.1, during the course of a murine infection generally concluded that CcpA is critical to virulence gene regulation inside mature lesions, whereas LacD.1 appears more important at early and late timepoints. These differences were perhaps mediated by the regulators sensing and responding to different environmental signals, such as glucose, during the course of infection, and may also be influenced by the site of infection (129). Finally, the major 'stand-alone' regulator of *S. pyogenes* Mga structurally resembles the transcriptional regulator MtlR of *G. stearothermophilus*, yet *S. pyogenes* possesses a CcpA-binding site upstream of the promoter for *mga* (130, 131). Studies of the PRD domain of Mga in a mouse model indicated that maintenance of its phosphorylation level is critical to virulence expression, further illustrating the importance of nutrient source and availability during the course of an active infection (131). Collectively, these studies demonstrate the complexity of metabolite-sensing regulatory circuits during *in vivo* infections and raise important questions of how metabolite sensitive regulators are coordinated during various phases of infection and disease.

S. pneumoniae is another major human pathogen that colonizes the pharynx and is often cleared asymptomatically, but can cause more severe disease in some cases. As *S. pneumoniae* moves away from the oral cavity and ventures into the airways, it is likely that this pathogen must liberate and metabolize nutrients from host glycoconjugates, including host defense molecules (132). In addition to a complement of glycosidases, a recent comprehensive study across several strains of *S. pneumoniae* has identified as many as 32 carbon sources directly metabolizable by this bacterium, with the vast majority being transported by PTS permeases (133). Of interest,

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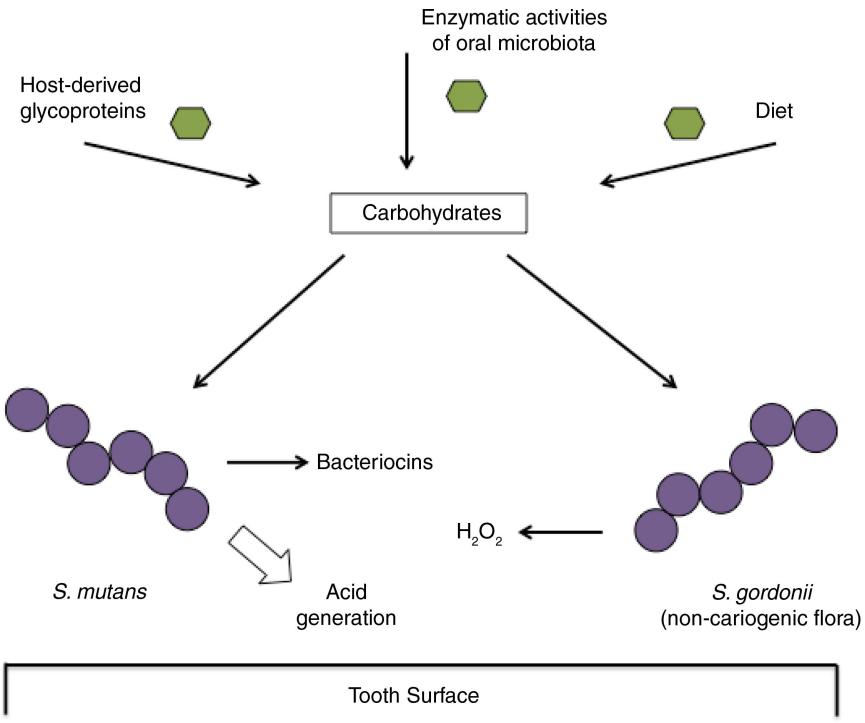

Fig. 2. Illustration of the response of *S. mutans* and non-cariogenic flora to carbohydrates introduced to the oral cavity.

this study demonstrated the importance of a mannose-type PTS transporter in the internalization of glucose, galactose, mannose, GlcNAc, and GlcN (133), similar to what has been seen in *S. mutans* (25, 107, 134); though, it was notable that the operon in *S. pneumoniae* lacks the accessory gene *manO* (133). As demonstrated in other streptococcal species, the success of *S. pneumoniae* within an *in vivo* infection model is greatly diminished in the absence of the major catabolite regulator CcpA (135, 136). The prominent influence of CcpA as a master regulator within *S. pneumoniae* was recently demonstrated in a study using transposon sequencing (Tn-seq), which implicated CcpA in 64 genetic interactions (137), and another study combining transcriptomic and metabolic analyses (138). Collectively, these recent advancements in our understanding of the human pathogens *S. pyogenes* and *S. pneumoniae* are beginning to reveal the critical role of carbohydrate uptake and related gene regulation during the course of infections.

Conclusions and future direction

For pathogenic streptococci, acquisition of nutrients is a key event in the survival within and exploitation of the host. It is readily apparent that oral cariogenic bacteria are a product of the dynamic environment that they inhabit and differences in the levels and types of carbohydrates presented to cariogenic species have a great influence on the caries process. The ability of *S. mutans* to coordinate carbohydrate metabolism in response to changing environmental factors is critical to its ability to outcompete other members of the oral microbiome when conditions are conducive for caries development (Fig. 2). Further, carbohydrate sources have been demonstrated to impact the ability of the pathogen to antagonize commensals via the production of bacteriocins (139) and conversely, the capacity of commensals to respond through the production of defensive compounds, such as hydrogen peroxide (140, 141). While the numerous and often complex interactions of *S. mutans* with other members of the oral microbiome undoubtedly impact carbohydrate utilization and its regulation, the many studies of the impact of carbohydrate availability on *S. mutans* in pure cultures have greatly enhanced our understanding of how phenotypic and genotypic events converge and provided a model for more complex, *in vivo* studies of this major pathogen. As tools evolve to study whole populations at the transcriptomic and metabolomic level, it will be a fascinating journey to understand how *S. mutans* and other abundant plaque streptococci thrive in the human host.

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Conflict of interest and funding

There is no conflict of interest in the present study for any of the authors.

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